Early Journal Content on JSTOR, Free to Anyone in the World

This article is one of nearly 500,000 scholarly works digitized and made freely available to everyone in the world by JSTOR.

Known as the Early Journal Content, this set of works include research articles, news, letters, and other writings published in more than 200 of the oldest leading academic journals. The works date from the mid-seventeenth to the early twentieth centuries.

We encourage people to read and share the Early Journal Content openly and to tell others that this resource exists. People may post this content online or redistribute in any way for non-commercial purposes.

Read more about Early Journal Content at http://about.jstor.org/participate-jstor/individuals/early-journal-content.

JSTOR is a digital library of academic journals, books, and primary source objects. JSTOR helps people discover, use, and build upon a wide range of content through a powerful research and teaching platform, and preserves this content for future generations. JSTOR is part of ITHAKA, a not-for-profit organization that also includes Ithaka S+R and Portico. For more information about JSTOR, please contact support@jstor.org.

COMPLEMENT FIXATION WITH ACID-FAST BACTERIA

I. A STUDY OF VARIOUS ORGANISMS WITH IMMUNE RABBIT SERUMS

J. V. Cooke

From the Department of Pathology and Bacteriology of the University of California, Berkeley, and the Department of Bacteriology, College of Physicians and Surgeons, Columbia University, New York

Since the discovery of the fixation reaction by Bordet and Gengou in 1901, there has been a lively interest in the application of the test to the recognition of specific antibodies to acid-fast bacteria, particularly the organisms causing tuberculosis and leprosy. It was while studying fixation in tuberculosis that Wassermann and Bruck in 1905 devised their well-known method for the serodiagnosis of syphilis. Following this discovery, the attention of workers in serology was largely taken up by the study of the Wassermann reaction so that several years elapsed before complement fixation with acid-fast organisms again became the object of much interest. Within the past few years, however, many workers have been experimenting with various tubercle antigens until at the present time the fixation reaction in tuberculosis, in the hands of several workers at least, is apparently specific and of considerable value in diagnosis.

Obviously much of the importance of complement fixation with bacterial antigens depends on the specificity of the antigens. With acid-fast antigens there has been much confusion, especially in the interpretation of results, because the specificity of such reactions is obscured by a rather widespread "group reaction." It has not been possible to distinguish various members of this group from each other with immune serums except in a very inconclusive and unsatisfactory manner. This group reaction has been assumed to be due to some antigenic substance common to the various acid-fast bacteria. For example, the serum of cattle with chronic paratuberculous enteritis (Johne's disease) will react by the fixation test almost equally well with antigens of the causative acid-fast bacillus and with human, bovine and avian tubercle bacilli. The lack of a clear-cut specificity is further illustrated by the fact that much of the earlier work on complement fixation in tuberculosis was hampered by the discovery that

Received for publication July 25, 1919.

many cases of syphilis without evidence of tuberculous infection gave positive fixation with tubercle bacilli and tuberculin antigens. Many observers have noted also that a number of cases of leprosy give fixations with various lipoidal tissue extract antigens used in the Wassermann test.

That the difficulties in the correct interpretation of the results of fixation reactions with acid-fast antigens are not fully appreciated by some observers may be illustrated by reference to the acid-fast bacilli that have been described and grown by numerous investigators from lepers. The organisms isolated by different observers have not been identical, and, since none has fulfilled Koch's requirements, the artificial cultivation of the true Hansen bacillus is not generally accepted as proved. Several of the acid-fast organisms have been used as antigens in fixation tests, and on finding fixation with leper serum it has been assumed that this was a specific reaction. In view of the group reaction, the attempt to justify the claim of the identity of any acid-fast organism with the Hansen bacillus by such indirect evidence is very unconvincing.

The entire question of complement fixation with acid-fast antigens, therefore, although not well understood, is one of considerable importance, and it is only by careful studies of the serum of immune animals, as well as the serum of patients suffering from various infections, that the value of these reactions can be determined and their specificity properly estimated.

A review of the experimental work in which acid-fast bacteria have been used as antigens, emphasizes the difficulty in interpretation of the results of fixation with this group in so far as specificity is concerned. While a large number of studies have been made with single organisms, especially tubercle bacilli, the work in which several acid fast bacilli have been compared antigenically is confined to relatively few papers which will be reviewed in the following paragraphs.

Gengou[1] immunized guinea-pigs against acid-fast organisms: the butter bacillus of Rabinowitsch (2 strains), of horse dung, timothy hay, Korn I, Tobler II, Tobler V, fish tuberculosis, blindworm tuberculosis, Arloing's bacillus of homogeneous tuberculosis, human, bovine and avian tubercle bacilli. The organisms were grown on glycerol potato, dried over KOH, powdered and suspended in salt solution. Immunization was carried out by 3 subcutaneous injections of 5 mg. each and the animals bled from 14 to 21 days after the last injection. Not all possible cross fixations were done except with the serum of the animals immunized against human tuberculosis. The immune serums of human, avian and bovine tuberculosis showed fixation with almost all the antigens used, while the cross fixations with other serums and acid-fast antigens were positive with few exceptions. A normal guinea-pig serum gave fixation with the timothy hay antigen so that it may have been anti-complementary. He concludes that the serum of guinea-pigs injected with acid-fast bacilli form sensitizers, not only against homologous bacilli, but also against the other acid-fast bacilli, especially human, bovine, and avian tuberculosis. This rule presents only rare exceptions.[2]

---

[1] Berl. klin. Wchnschr., 1906, 43, p. 1531.

Much and Hoessli[3] used as antigens washed bacillary emulsions of organisms grown in broth, ground and suspended in phenolized salt solution. The strains studied were bacillus of human tuberculosis, urine bacilli (smegma?), blindworm bacilli and bacillus of timothy hay. Of serums from 100 cases of proved tuberculosis, 30 reacted positively with the tubercle bacillus. Of these, 19 were tested with the 3 other antigens and 13 reacted positively with urine bacilli, 4 with blindworm antigen, while none gave fixation with timothy hay bacillus. In addition, the serums of two rabbits immunized with urine bacilli gave fixation with urine bacilli, blindworm bacilli and human tubercle bacilli and also with tuberculin and a broth filtrate of blindworm bacilli. Controls using broth and peptone solution as antigen were negative.

Much and Leschke,[4] using the serums of two goats injected with human tubercle bacilli, got fixation with emulsions of human tubercle bacilli, urine bacilli, blindworm bacilli, timothy hay bacilli, leprosy bacilli (from antiforminized leprous skin), tuberculin and urine bacilli broth filtrate, but negative tests with blindworm bacilli broth filtrate and plain broth.

Deilmann[5] studied human serum with emulsions of tubercle bacilli, urine bacilli, timothy hay bacilli, blindworm bacilli, leprosy bacilli (from antiforminized nodules) and tuberculin. Complement fixations were made in 239 persons ill with various diseases. In a large percentage of these cases there was no clinical tuberculosis nor tuberculous history. About 50% (118) reacted to one or more of the antigens used and of the positive reactions 84.6% were with tuberculin. With tubercle bacilli emulsion only 69.5% reacted positively. A few cases (15) were tested with lepra bacilli and 33% gave positive fixations. Many of the cases were tried with the blindworm, timothy hay and urine bacilli antigens, and small percentages of positive reactions were found although six cases gave reactions with urine bacilli and not with either of the tubercle antigens. Of the 118 cases in the series that gave positive reactions, only 11 were clinically tuberculous. He concludes that the tubercle bacillus contains specific complement binding substances in common with other related acid-fast bacilli. He also states that the complement binding substances present in human serum against tubercle antigen have little relation to clinically demonstrable tuberculosis but only indicate a previous contact tuberculosis. Against this last conclusion two important objections may be urged: First, that he used no control non-acid-fast antigens, and second, that the relatively large amounts of antigen used in each test—from 0.66 to 3.3 mg.—might lead to non-specific fixations, since control cases of other diseases were not included.

---

[2] Gengou has since privately stated that the potato medium employed in growing the antigens was probably itself antigenic and accounted for the cross reactions. (Information furnished by F. P. Gay.)

[3] Beitr. z. Klin. d. Tuberk., 1910, 17, p. 199.

[4] Ibid., 1911, 20, p. 351.

[5] Ztschr. f. Immunitätsforsch. u. exper. Therap. (Orig.), 1911, 10, p. 421.

Using the same antigens as Deilmann, Wills[6] tested 18 serums that had given positive reactions with the tuberculin antigen and got positive fixations with tubercle bacilli in 83%, leprosy bacilli in 27%, urine bacilli in 27%, timothy hay bacilli in 11%, and blindworm bacilli in 5%. All the antigens, with bovine tubercle bacillary emulsions also, gave positive fixation in more than half of 11 cases of leprosy tested.

Edith Claypole,[7] in her classification of the streptothrix group of bacteria on the basis of fixation, found not only group reactions between acid-fast members of the streptothrices, but between them and the tubercle bacillus on the one hand and the non-acid fast streptothrices on the other. By means of variable amounts of antigen, however, relative differences between homologous and heterologous strains and a given immune serum could be shown. This work of Claypole's and her method formed the point of departure in our own study to be herewith presented.

Kritchewsky and Bierger[8] report that the serums of a rabbit and a monkey immunized with Kedrowsky's leprosy bacillus gave fixations with human tubercle bacilli and Duval's chromogenic leprosy culture somewhat weaker than the homologous antigen. Antigens of Korn's bacillus I and B. typhosus did not react with the serum. Tests of four rabbits injected with Kedrowsky's culture were negative with Duval's culture although giving good fixation with the Kedrowsky antigen. One of these serums when tested with human tubercle bacilli gave a positive reaction. Two other rabbits immunized with Duval's culture, while reacting well with the homologous antigen, were negative with Kedrowsky's[9] culture.

Harris and Lanford[10] immunized rabbits against B. smegmatis, timothy hay bacillus, B. tuberculosis (avian) and the acid-fast organisms isolated from lepers by Kedrowsky, Bayon, Clegg, Currie and Duval (chromogenic and nonchromogenic strains). They tested these serums with antigens made from these organisms, and the butter bacillus and dung bacillus. In addition the serum of a rabbit injected with bacilli from a leper nodule was used in some of the tests. The organisms were grown on glycerol agar and the antigens used were 2% emulsions in salt solution of the alcohol precipitated bacterial growth. With decreasing doses of the various antigens in cross fixations until the minimal fixing dose was reached, an attempt was made to differentiate the several organisms. Cross fixation, however, was almost universal, and it was not uncommon that the minimal fixing dose of homologous antigen with an antiserum was higher than the minimal fixing dose of heterologous antigen. They concluded that their fixation tests showed no clear-cut specificity for any of the acid-fast bacilli studied.

Although these reports of previous investigators show the presence of a well-marked group reaction, including many of the acid-fast organisms, with consequent difficulty in classifying this group of organisms by complement fixation, a further attempt to use this method as a basis of grouping has been made. The first series of tests differed considerably from later experiments, and will be considered separately.

---

[6] Centralbl. f. Bakteriol., O., I, 1912, 61, p. 37.

[7] Jour. Exper. Med., 1913, 17, p. 99.

[8] Ztschr. f. Hyg. u. Infectionskrankh., 1913, 73, p. 509.

[9] Ibid., 1901, 37, p. 52; 1910, 66, p. 1.

[10] J. Infect. Dis., 1913, 13, p. 301.

METHODS

Antigens.—The acid-fast bacilli used were cultures of the following organisms: the strains of B. leprae of Duval[11] (chromogenic and nonchromogenic), of Bayon, of Brinckerhoff,[12] of Kedrowsky,[9] of Clegg,[13] of Levy[14] and Karlinsky;[15] B. tuberculosis, human and bovine types; B. smegmatis, butter bacillus (Rabinowitsch), and dung bacillus (Moeller No. 1), and B. Lombardo Pellegrino[16] (a pigmented saprophyte). All cultures except those of B. tuberculosis were obtained from Dr. C. W. Duval. Duval's[11] nonchromogenic strain of B. leprae was at first grown with difficulty but after several transplants grew well on glycerol agar. The cultures of B. tuberculosis were transplants from strains originally obtained from Dr. Theobald Smith.

A discussion of the growth characteristics and morphology of each of these bacilli will not be given here. The various organisms differed in their degree of "fastness" when treated with acids or with alcohol as all workers have found. The strain of B. smegmatis conformed to the usual description by being acid-fast but not alcohol fast.

The tubercle bacilli were cultivated on Dorset's egg medium for from 4 to 6 weeks. The remainder were grown on glycerol agar in large flat Blake bottles. When the growth was fairly abundant, which was at the end of from one to two weeks, it was scraped off and washed from the surface of the medium with as little salt solution as necessary. This bacillary emulsion was then treated with somewhat more than an equal volume of 95% alcohol when the protein was precipitated as a flocculent precipitate. After immediate rapid centrifugalization and the removal of the clear supernatant fluid, the bacteria were dried over sulphuric acid in partial vacuum and later pulverized. This bacterial powder was finely ground in an agate mortar and a 2% suspension in 0.85% salt solution with 0.5% phenol. These 2% bacterial suspensions were used as antigens.

In the tests a constant amount of serum was used, and progressively decreasing doses of antigen dilutions were made with 0.85% salt solution. One c c of 1:20, 1:40, 1:80, 1:160, etc., dilutions of the antigens representing 1, 0.5, 0.25, 0.125 mg. etc., respectively, of the dried bacterial protein were used.

Immune Serum.—Two rabbits were immunized with each antigen by 5 intravenous injections at 3-day intervals and bled 9 days after the last injection. Each intravenous injection was 0.5 c c of the antigen, i. e., 10 mg. of dried bacterial powder. The immune serum was inactivated 30 minutes at 56 C. and kept in sealed glass tubes. In each test 0.3 c c were used. Of the two serums obtained, the one which showed fixation with the highest dilution of the homologous antigen was used for the tests.

Hemolytic System.—This consisted of 4 units of a rabbit antisheep serum of 1:5,000 titer, 1 c c of a 5% suspension of sheep corpuscles and a fixed dose of 0.1 c c of fresh guinea-pig serum.

The test was made by incubating the immune serum and antigen with fresh solution at 37 C. for 1 hour in the water bath, adding the lysin and sheep cells, incubating again for 2 hours, and reading the results after allowing the tubes to stand over night in the icebox. The total volume of the test was 2.5 c c. The usual controls for anticomplementary properties in antigen and serum were always used, and tests with every antigen and normal rabbit serum showed no nonspecific fixation in the dilutions used.

---

[11] J. Exper. Med., 1910, 12, p. 649; J. Infect. Dis., 1912, 11, p. 116.

[12] Pub. Health Rep., U. S. Mar. Hosp. Serv., 1910, 25, p. 1173.

[13] Philippine J. Sc., 1909, 4, p. 403.

[14] Gior. d. r. Soc. ital. d'ig., 1902, 24, p. 219.

[15] Monatsh. f. prakt. Dermat., 1903, 37, p. 392; Allg. med. Centr.-Ztg., 1903, 72, p. 965.

[16] Ann. d'ig. Sper., 1906, n. s., 16, p. 163.

In testing a given immune serum successive decreasing doses of homologous antigen were used with a constant amount of serum until the smallest amount of antigen that gave fixation was reached. Cross fixations were then made with this serum and the other antigens in the same manner. Each immune serum, therefore, was used in cross fixations with descending doses of all antigens. In reading the results of the tests, considerable difficulty was encountered in determining the point at which sufficient inhibition occurred in a series of antigen dilutions to call the test positive. When larger amounts of antigen were employed there would be complete inhibition and as less and less antigen was used hemolysis would gradually increase until finally it was complete. For example, in testing a given serum with 1:20 and 1:40 dilution of antigen no hemolysis would result, in the 1:80 and 1:160 dilutions, hemolysis would be partial and in 1:320 dilution it would be almost complete. On account of these gradations, the readings were always made at the lowest dilution of antigen which showed complete inhibition of hemolysis. The anticomplementary properties of the different antigens were carefully determined and no fixation test was considered positive unless double the dose of antigen used was not at all anticomplementary.

Table 1 shows the results of the fixation with the various antigens and immune rabbits serums.

It must be remembered that the readings were made at the point where there was complete inhibition of hemolysis, and that those reactions marked "o" in the table do not mean that there was no evidence of cross-fixation, but that the fixation was not complete with half the antigen dose that was not at all anticomplementary. Indeed, there was in all these instances very definite partial cross-fixation. Several of the serums, including those of animals immunized with tubercle bacilli, were tested with only a few of the antigens. There is no indication, however, that the completion of the cross-fixations with these serums would have altered the general results obtained.

In studying the tabulated results of the tests it is quite evident that there is a widespread group reaction that includes all the acid-fast organisms studied and that any classification or grouping of the strains cannot be made by the use of the methods employed in this series. It will be noted that the B. leprae cultures of Duval (chromogenic) and of Levy, showed fixation in greater dilutions with the immune serums of these two organisms than with any of the other serums. This same grouping is also noted with Duval's[11] nonchromogenic strain and Kedrowsky's[9] culture. The other cross-fixations reveal diffierences so slight and inconsistent that any effort at grouping based on these results

TABLE 1

Complement Fixation with Immune Rabbit Serums Showing Dilutions of Antigens Giving Complete Fixation with Fixed Dose of 0.3 c c of Immune Rabbit Serums

Antigens (2%)	Largest Amount of Antigen Not Anticomplementary (Antigen Control)	Immune Rabbit Serums (0.3 c c)														
		Normal Serum Control	B. leprae Duval (Chrome)	B. leprae Bayon	B. leprae Brinckerhoff	B. leprae Clegg	B. leprae Levy	B. leprae Duval (Nonchrome)	B. leprae Kedrowsky	B. leprae Karlinsky	B. smegmatis	B. of Dung Moeller	B. of Butter	B. Lombardo Pellegrino	B. tuberculosis (Human)	B. tuberculosis (Bovine)
B. leprae—Duval (chrome)	1-10	+	**1-320**	1-20	0	0	1-160	1-20	0	—	0	1-20	0	1-40	—	—
B. leprae—Bayon	1-5	+	1-20	**1-80**	1-20	1-20	1-20	1-20	1-20	—	1-40	—	1-20	1-80	—	—
B. leprae—Brinckerhoff	1-3	+	1-20	1-20	**1-80**	1-80	1-40	1-20	1-20	—	1-40	—	1-20	1-80	—	—
B. leprae—Clegg	1-5	+	1-20	1-20	1-20	**1-40**	1-20	1-20	1-20	—	1-40	—	1-80	1-160	—	—
B. leprae—Levy	1-3	+	1-320	1-20	0	1-10	**1-160**	1-20	0	—	1-40	—	1-10	1-40	1-20	—
B. leprae—Duval (nonchrome)	1-10	+	1-40	1-40	1-40	1-40	1-40	**1-80**	1-80	—	1-40	—	1-40	1-40	—	—
B. leprae—Kedrowsky	1-10	+	1-40	1-40	1-20	1-20	1-40	1-80	**1-80**	—	1-40	—	1-40	1-40	1-40	—
B. leprae—Karlinsky	1-3	+	1-20	1-40	1-10	1-10	1-20	1-10	1-20	**1-20**	—	—	1-40	1-20	—	—
B. smegmatis	1-10	+	1-80	1-80	1-80	1-40	1-40	1-40	1-40	—	**1-320**	—	1-160	1-40	—	—
B. of dung—Moeller No. 1	1-5	+	1-20	1-40	1-10	1-10	1-10	1-20	1-10	—	1-20	**1-40**	1-20	1-80	—	—
B. of butter—Rabinowitsch	1-3	+	1-20	1-20	1-10	1-10	1-20	1-20	1-10	—	—	—	**1-40**	1-20	—	—
B. Lombardo Pellegrino	1-10	+	1-20	1-20	0	0	0	0	0	—	1-20	—	—	**1-160**	—	—
B. tuberculosis—human	1-5	+	—	1-10	1-10	1-10	1-10	1-20	1-20	—	—	—	1-10	1-10	**1-40**	1-20
B. tuberculosis—bovine	1-3	+	—	1-10	0	0	1-10	1-20	1-20	—	—	—	1-10	0	1-20	**1-20**
No antigen (serum control)	—	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

1-1, 1-20, 1-40, etc., indicate 1 c c of the corresponding dilution of the stock 2% antigen; + shows complete hemolysis; 0 a negative result and — the test not performed.

would not be justified. Occasionally an immune serum will fix a heterologous antigen in greater dilution than the homologous antigen. Since certain important points relating to the cross-fixations by different members of this group were demonstrated in other tests, a more detailed discussion of this subject will be made later. One feature, however, may be mentioned here. In making the antigens from the dried bacterial powder, a considerable variation in the evenness of the emulsion was observed. Some of the strains gave an antigen in which the emulsion was composed of a uniform and apparently even suspension of bacterial powder in a fine state of division, while other antigens showed some coarser particles. The coarser particles could not be overcome by long, continued grinding of the powder in an agate mortar, and were probably due in some way to the fatty substances of these particular organisms. The importance of this variation in the antigen emulsions lies in the fact that the antigens which gave the best emulsification with salt solution were those which gave fixation in the greatest dilution with the various immune serums, while those antigens in which the particles were coarser showed fixation only in low dilutions. There was, therefore, a definite variation in the antigenic properties of the different antigens due to the fact that all could not be emulsified equally well in salt solution. This factor naturally interferes with any deductions drawn from the comparison of the amounts of the different antigens necessary for complement fixation with immune serums.

In examining the results obtained by this series of fixations, it seemed that the method of study used was open to several objections, or at least that certain rather fundamental improvements in the methods used would overcome some of the difficulties in the interpretation of the results. The determination of the least amount of antigen necessary to give fixation with a given serum, for example, is merely an antigen titration and does not necessarily indicate the concentration of complement-fixing substances in the serum. It would seem of much more value to titrate serum with a fixed dose of antigen and thereby determine the least amount of serum that would react with the various antigens. It would also be of advantage to test the specificity of the reaction by control cross-fixations with organisms of this group and unrelated nonacid-fast organisms. It is obvious that with such a widespread group reaction as shown in this series, a more delicate hemolytic system would contrast the reactions with different antigens more sharply and offer a better chance for differentiation. Since the binding of a certain amount of complement is what constitutes a positive reaction, and the less fixation substances are present in a serum the less complement will be fixed, the test can readily be made more delicate by using as small an amount of complement as can be employed with safety. Also, the difficulty experienced in interpreting fixations that are incomplete can be greatly obviated by employing a weaker suspension of red cells in the hemolytic system. In all later work the methods used were altered and the improvements suggested by this first series were made the basis for a further study of fixation reactions with this group.

In the second series of experiments, three important variations were introduced as a result of the difficulties in the first series. These were: First, the use of a larger number of acid-fast bacterial antigens with the addition of several nonacid-fast organisms as controls; second, the titration of the concentration of the fixation antibodies in all immune serums with each of the antigens; and third, the use of a more delicate and accurately controlled hemolytic system in which relatively small amounts of complement were necessary. By the use of these methods, the experiments were considerably broadened, and several interesting features concerning fixation with immune rabbit serums and bacterial antigens, especially those belonging to the acid-fast group, have been studied.

Antigens.—The acid-fast organisms used as antigens included 11 strains isolated from lepers, the B. leprae of Duval[11] (chromogenic and nonchromogenic), of Levy,[14] of Kedrowsky,[9] of Karlinsky,[15] of Bayon, and the cultures B, F, G, H and 18, isolated by Clegg, Currie, Brinkerhoff and Hollman;[12, 13, 17] 4 cultures of saprophytic acid-fasts, smegma bacillus, butter bacillus (Rabinowitsch), the dung and timothy bacillus of Moeller;[18] 4 cultures of B. tuberculosis, human, bovine, avian and the "turtle" culture of Friedmann. The nonacid-fast cultures used as control antigens were B. typhosus (Rawlings strain), B. coli, B. abortus, and a diphtheroid bacillus isolated from a lymph node from a case of Hodgkin's disease. In addition, an emulsion of Hansen bacilli from lepromas was used as an antigen. The strains of B. leprae isolated by Clegg and his co-workers were obtained from the late Dr. D. H. Currie, the Friedmann culture from the New York Museum of Natural History, the strain of avian tuberculosis (Avian 216) from Dr. Krumweide of the New York Department of Health, the human and bovine tubercle bacilli from Dr. Harry Foster of the Cutter Laboratories, Berkeley, Calif., and the remainder of the acid-fast organisms from Dr. C. W. Duval of New Orleans.

[17] Treas. Dept. Bull. 47, Pub. Health and Mar. Hosp. Serv. 1911; Lepra, 1912, 13, p. 71.

[18] Deutsche med. Wchnschr., 1898, 24, p. 376.

The bacterial antigens were prepared in the same manner as those used in the previous series. Human and bovine tubercle bacilli were grown on Dorset's egg medium, the other acid-fast bacilli on glycerol agar and the non-acid-fast cultures on plain agar. The dried, powdered alcoholic precipitate of bacterial culture was prepared as described. Emulsions of these antigens were made in salt solution so that one c c of the emulsion was equivalent to one mg. of the dried bacterial powder.

The material containing the leprous nodules was obtained at necropsy in a case of advanced nodular leprosy. Several portions of thickened skin containing large numbers of leprosy bacilli were cut in small pieces and digested with antiformin at 37 C. until the tissue was disintegrated. It was then centrifuged and the sediment washed several times in salt soluion. The sediment had a definitely yellow color and microscopically consisted entirely of acid-fast bacilli. It was dried over sulphuric acid and the bacterial powder used as antigen in exactly the same manner as the organisms from cultures.

In preparing antigen for use, 20 to 30 mg. of the powder were weighed out and thoroughly ground in an agate mortar. A more even emulsion was obtained by moistening the powder with salt solution and thoroughly mixing by grinding as the additional amount of salt solution necessary to make one mg. per c c was added. This antigen emulsion was used both for immunization of animals and for the fixation tests. It was kept at low temperature when not in use and frozen if possible. The addition of phenol or tricresol as a preservative perceptibly increased the anticomplementary action so that no preservative was used and freezing was depended on to prevent contaminating bacterial growth. The anticomplementary powers of such emulsions increased very gradually and after several weeks it was necessary to prepare a new antigen emulsion from the dried powder. The anticomplementary dose of such antigens varied from 0.2 to 2 c c (or mg.) and was relatively constant in freshly prepared emulsions of any antigen.

Immune Serums.—Two rabbits of 1,800 to 3,500 gm. were immunized with each antigen. Before immunization the serum of each animal was tested for nonspecific fixation with the antigen and if obtained with less than 0.4 c c of serum, the animal was discarded and another used. Four intravenous injections of from 1 to 4 mg. were given at 3 day intervals and 3 days after the last injection a final intraperitoneal dose of 6 to 12 mg. The size of the dose was determined by the weight reaction of the animal to previous doses. Animals were bled from 9 to 11 days after the intraperitoneal injection. The serum was sealed in small tubes and inactivated at 56 C. For the cross-fixations with other antigens, only the serum having the highest titer with the homologous antigen was used.

The Hemolytic System.—Various forms of hemolytic systems have been used in experimental work but the technic used in these experiments has proved so satisfactory in many ways that it is given in slightly greater detail than is usually thought necessary. No claim is made for originating it, however, since others have probably used a very similar procedure. Its use in a very large number of tests has provevd its reliability and its delicacy.

The system consists of 2 units of complement, with 2 units of hemolytic sensitizer and 0.5 c c of a 1% suspension of sheep corpuscles made up to a total volume of 2.5 c c. The various ingredients must be standardized carefully.

The 1% suspension of sheep cells is more satisfactory when measured by centrifuging in a graduated centrifuge tube rather than by a pipet. The sediment should be lightly packed.

Rabbit antisheep serum of moderately high titer is diluted so that one minimal hemolytic dose with 1 c c of 1% dilution of complement is contained in 0.1 c c.

Pooled fresh guinea-pig serum is diluted 1:100. The complement unit is usually found to be 0.5 c c of this 1% dilution.

A preliminary titration of hemolysin with 2 units of complement, and of complement with 2 units of hemolysin is made as follows: Tubes are set up with 0.2, 0.15, 0.1 and 0.05 c c of hemolysin with 1 c c of complement; 0.8, 0.6, 0.5 and 0.4 of complement with 0.2 c c of hemolysin. All tubes are made up to 2 c c and 0.5 c c of 1% sheep cells added. The usual controls of hemolysin, complement and cells alone are included. Readings are made after one-half hour in the water bath at 37 C. In the hemolysin titration all tubes should be completely laked except the one containing only 0.05 c c of the hemolysin in which the hemolysis is incomplete. The complement titration should show complete laking in the first 3 tubes, leaving the tube with only 0.4 of complement partially hemolyzed. The controls should show no hemolysis.

It is rather remarkable how constantly the unit of complement is found to be 0.5 c c in this titration whether serum from one guinea-pig or pooled serum from several animals is used, provided the serum is fresh. The only factor that may cause some variation is the corpuscle suspension. In case the corpuscle emulsion is considerably more than 1%—as, for example, when it is made up from a very tightly packed centrifuge sediment—the complement titration will show a unit of 0.6 or 0.7 c c. In this case the usual values will be found by a slight dilution of the red cell suspension. It is almost unnecessary to say that the same error will follow the use of partially hemolyzed blood containing broken-down cells. A much more important factor, however, is the variation in resistance of the red cells of different sheep, since the corpuscles of some animals are very definitely more resistant to the hemolysis than the cells of other animals. In all the work here reported, the corpuscles used were obtained from animals kept by the laboratory and trouble from this source was not encountered since animals whose corpuscles were resistant were discarded. More recently, however, in doing similar tests it has been necessary to use blood obtained from abattoirs and the finding of cells somewhat more resistant to hemolysis has been not infrequent. With such blood the titration seems to indicate a weak or poorly acting complement, but it is relatively easy to demonstrate that the fault practically always lies entirely with the corpuscles. In such a case, if other less resistant cells are not available, one can only accept the unit shown in the titration.

The Tests.—The inactivated serum from an animal immunized with one of the antigens was tested as follows: Gradually decreasing amounts of serum from 0.05 c c to 0.0001 c c were measured into a series of tubes. For convenience and accuracy this was measured from dilutions of 1:10, 1:100 and 1:1,000. The antigen which had been previously titrated to determine the anticomplementary dose, was then added in less than one-half the dose that was not at all anticomplementary. As a rule, the amount used was 0.1 c c which corresponds to 0.1 mg. of the bacterial powder, and in all cases from 4 to 8 times this amount was not anticomplementary. After adding two units of complement (1 c c of a 1% dilution) and sufficient salt solution to make a total of 1.8 c c, the tubes were incubated in the water bath at 37 C. At the end of one hour 0.5 c c of 1% sheep cells sensitized with two units of hemolytic serum (0.2 c c) was added and the tests incubated one-half hour longer. Readings were made after standing over night in the icebox. The usual controls of antigen alone and serum alone were always added to each series. Such a titration was made with every immune serum, not only with its corresponding antigen, but with all the antigens used.

In reading the tests the point at which complete inhibition ceased was always fairly sharply defined, and little effort was made to distinguish degrees of hemolysis. The standard adopted for reading titrations was complete inhibition or a very faint tinging of the supernatent fluid. The amount of serum in the last tube showing such an appearance indicated the minimal fixing dose of the serum with that antigen. The point at which inhibition stopped was so sharply defined that the result was never in doubt and the personal equation in reading the tests can be disregarded.

Time of Fixation.—The optimum time for the fixations was determined by finding the time at which the minimum amount of serum would give complete fixation with antigen; or, in other words, by finding the optimum incubation necessary for recognition of the smallest amount of complement-fixing antibody with antigen. For this purpose, the minimum fixing dose of serum with antigen was determined after incubating separate sets of tubes in the water bath at 37 C. for periods of 30, 45, 60 and 75 minutes; in the air bath for the same periods; and at 8 C. (icebox) for 4 hours. It was found that smaller amounts of serum gave fixation after water-bath incubation than after air-incubation during the same period. Almost always tubes incubated in the water bath for one hour showed a higher titer after one hour than for shorter periods, and this was not increased by leaving them 15 minutes longer. Incubation in the icebox (8 C.) for 4 hours showed also somewhat lower titer than after one hour in the water bath. Observations on a number of serums with several antigens constantly confirmed the fact that one hour in the water bath at 37 C. was the optimum incubation of those studied and consequently this period was adopted for all the tests.

Amount of Antigen Used in Tests.—By using the dried bacterial powder prepared as described, it was possible to use constant and comparable amounts of antigen in tests carried out at various times with the same or different immune serums. The question of the proper amount of antigen to use in the test was one which required some study. Two points in particular seemed necessary to determine: first, whether an increased amount of antigen above several minimal fixing units would increase the sensitiveness of the fixation test, i. e., if one has several times the amount of antigen sufficient to combine with a fixed amount of alexin and a certain quantity of fixation antibody, will an increase in the amount of antigen cause fixation with a still smaller quantity of immune body? Second, whether a relatively large amount of antigen has a tendency to cause a nonspecific fixation independent of any true anticomplementary action. The first of these two points was determined by finding the minimal fixing dose of an immune serum with an arbitrary dose of antigen, e. g., 0.1 mg. With three times this amount of serum, i. e., 3 minimal fixing doses, the minimal fixing dose of antigen, or antigenic unit, was titrated, and finally the serum was again titrated and the minimal fixing dose again determined, using 3 antigenic units. In this manner several different serums and antigens were studied and the results were quite uniform in indicating that if at least 3 units of antigen are used in titrating a serum, fixation will be obtained with quite as small amounts of the serum as if 10 or 20 antigenic units are used. It seems unnecessary to give more than a single example of such tests. The serum of a monkey dead from tuberculosis was titrated with 0.2 mg. of human tubercle bacilli antigen (of which 1 mg. was not anticomplementary) and the minimal fixing dose of serum found to be 0.004 c c.

Using a fixed dose of 0.012 c c of serum, the antigen was titrated and the minimum fixing dose of antigen or antigenic unit was determined as 0.01 mg. A retitration of the serum using 0.03 mg. of antigen was identical with the original titration, the minimal fixing amount of serum being 0.004 c c.

The tendency of an unusually large amount of antigen to cause nonspecific fixation with the immune rabbit serums quite independently of any anticomplementary action has been definitely indicated in the course of the experiments. It is most noticeable when doing cross-fixations with highly immune serums and heterologous antigens, and for this reason care must always be exercised in the amount of bacterial antigen used. It is felt that the use of from 0.05 to 0.1 mg. of antigen in the tests here reported probably reduced this factor to a minimum. A more detailed discussion of this nonspecific fixation will be given later.

Controls.—In every series of tests the usual control tests for anticomplementary properties in the serums and in the antigens were set up using double the largest amount of these reagents alone. Every serum was shown to contain only a slight amount of natural hemolytic sensitizer. The fact that 0.4 c c of serum from each rabbit was tested before immunization and gave no fixation with antigen was considered a sufficient control for the absence of fixation with normal serum and a given antigen. A further control of normal rabbit serum with each series of tests was, therefore, omitted.

In order to illustrate the method by which the tests were made, the record of one typical immune rabbit serum will be given together with a table showing its reactions with several of the antigens. Other immune serums for each of the antigens were made and tested in the same manner. A later table summarizing the results of all the cross-fixations will be understood to have been compiled from a series of tests such as the one given as an example.

RABBIT 507.—Weight, 2,160 gm.; serum from a preliminary bleeding which was tested Nov. 13, 1916, with antigen B. leprae (Kedrowsky) showed no fixation in 0.4 c c. The rabbit was given intravenous injections of the same antigen as follows: Nov. 14, 3 mg.; Nov. 17, 20 and 23, 4 mg., and on Nov. 28, 12 mg. intraperitoneally. On Dec. 9, the 11th day after the intraperitoneal injection, the animal was exsanguinated, the serum collected, sealed in ampules and inactivated.

TABLE 2

TITRATION OF FIXATION ANTIBODIES WITH VARIOUS ANTIGENS — RABBIT 507

Antigen 0.1 mg.	Amounts of Serum in c c										Control Antigen 0.2 c c
	0.2	0.008	-0.006	0.004	0.002	0.001	0.0006	0.0004	0.0002	0.0001	
B. leprae—Kedrowsky	..	++	++	++	++	++	++	++	+	—	—
B. leprae—Duval (chrome)	..	++	+	±	—	—	—	—	—	—	—
B. leprae—F	..	++	++	++	—	—	—	—	—	—	—
B. leprae—H	..	++	+	±	—	—	—	—	—	—	—
B. tuberculosis—human	..	++	++	++	++	++	++	++	+	—	—
B. tuberculosis—avian	..	++	++	++	++	+	—	—	—	—	—
B. of butter	..	++	++	++	++	—	—	—	—	—	—
B. of timothy	..	++	++	++	±	—	—	—	—	—	—
No antigen	—										

++ = complete inhibition; + = very faint hemolysis; ± = almost complete hemolysis: — = complete hemolysis.

Table 2 shows the results of fixation tests made with this serum March 17, 1917. Only a few of the antigens are given, although cross-fixations were made with all antigens as may be noted by referring to the larger table in which the complete results are given. The method of recording the tests in table 2 is as follows: "+ +" denotes complete absence of hemolysis, "+" denotes almost complete inhibition with very faint hemoglobin-tinging of the supernatant fluid, "±" denotes marked but incomplete hemolysis, and "—" complete hemolysis. The relatively weak cell suspension made such readings very sharp after standing 12 hours in the icebox. Both "+ +" and "+" were considered positive, while "±" and "—" were called negative.

Before taking up the completed results of the tests, a few observations on the persistence of the fixation antibodies in rabbit serum may be mentioned. One animal after immunization with B. tuberculosis (human) showed a fixation with 0.0006 c c of serum with B. tuberculosis antigen. This animal was bled again two months later and the serum then showed fixation with 0.03 c c when tested with the same antigen. This indicates that while such antibodies may persist for a considerable time, the concentration becomes much weaker. In this respect, they resemble other specific immune substances. Other observations relate to the persistence of such fixation bodies in serum which has been kept sterile in sealed tubes for long periods. Samples of three serums, which had been used for tests in the first series of experiments mentioned in this paper and had been kept at room temperature, were retested with the second series, from 3½ to 4 years later. Unfortunately, the original titer of these serums cannot be stated since they were examined only by antigen titrations, but antibodies in rather high concentration persisted in these serums after this relatively long period. These serums were from rabbits immunized with antigens of the strains of B. leprae of Duval (chromogenic and nonchromogenic) and of Kedrowsky. They showed fixation titers of 0.006 c c, 0.001 c c and 0.006 c c, respectively, with Duval's[11] chrome, Duval's nonchrome, and Kedrowsky's[9] organisms, which were the homologous antigens. All three serums gave fixation with a number of other acid-fast antigens, but, as a rule, in somewhat larger amounts. This evident slow deterioration of such antibodies has some bearing on the present series of experiments. On account of the number of immune rabbit serums and antigens studied, it was not always possible to test each serum immediately and sometimes several months elapsed before all the cross-fixations were completed with a given serum. It was, therefore, gratifying to have evidence that any marked deterioration in the fixing properties of a serum was unlikely. The observations just cited, together with the tests in general, give evidence, also, that the dried bacterial powder retains its antigenic properties unimpaired for a number of years.

The results of all the cross-fixations are summarized in table 3, which gives the minimal fixing dose of each serum with the various antigens. For convenience, the serum amounts are expressed in cubic millimeters instead of cubic centimeters, e. g., 1 = 0.001 c c, 50 = 0.05 c c. A blank indicates that the test was not made and 0 means that 0.2 c c of serum gave no fixation. The other features need no explanation since they seem clearly indicated in the table.

At first glance, the cross fixation seems to be practically universal, not only with the acid-fast antigens, but also with the few nonacid-fast organisms studied. It will be noted, however, that, while a given serum may fix with a number of antigens, usually a relatively small

TABLE 3

Fixation Reactions with Immune Rabbit Serums and Acid-Fast Antigens; Minimal Fixing Dose of Each Serum Expressed in Cubic Millimeters

	Largest Amount of Antigen Not Inhibitory, in Mg.	Amount of Antigen Used in Each Test, in Mg.	Antigenic Unit in Mg.	Immune Rabbit Serum																						
				B. leprae—Duval (chrome)	B. leprae—Bayon	B. leprae—Levy	B. leprae—B	B. leprae—F	B. leprae—G	B. leprae—H	B. leprae—18	B. leprae—Hansen	B. leprae—Kedrowsky	B. leprae—Duval (nonchrome)	B. tuberculosis—Human	B. tuberculosis—Bovine	B. tuberculosis—Avian	B. tuberculosis—Turtle	B. of dung—Moeller	B. leprae—Karlinsky	B. of timothy—Moeller	B. of butter	B. smegmatis	B. typhosus	B. diphtheroid, Serum 1	B. diphtheroid, Serum 2
B. leprae—Duval (chrome)	0.8	0.1	0.006	**2**	2	4	6	30	30	30	30	10	6	6	2	1	4	1	30	6	2	4	30	8	..	..
B. leprae—Bayon	0.8	0.1	....	50	**0.6**	6	6	30	8	100	..	30	4	4	6	2	8	1	4	2	1	2	6	6	30	0
B. leprae—Levy	0.6	0.1	....	4	4	**1**	4	30	30	30	30	8	4	4	2	2	30	4	30	30	2	4	30	30	..	..
B. leprae—B	0.8	0.1	....	2	4	2	**1**	10	8	30	6	6	2	4	1	1	2	2	8	..	..	1	4	..	30	0
B. leprae—F	2.0	0.1	0.02	8	4	4	8	**1**	2	4	8	30	4	8	4	1	4	4	8	..	4	4	8	..	..	..
B. leprae—G	1.0	0.1	0.05	6	4	4	4	1	**1**	4	8	30	4	10	4	2	6	8	10	..	4	4	8	..	..	..
B. leprae—H	0.8	0.1	0.04	8	6	8	8	1	1	**2**	10	30	6	30	4	1	6	10	8	..	4	6	6	..	..	..
B. leprae—18	0.4	0.1	....	100	8	30	10	0	30	50	**4**	100	8	0	4	4	50	30	50	..	30	8	0	..	..	..
B. leprae—Hansen (from skin)	0.8	0.1	0.05	30	..	6	0	30	30	50	10	**4**	2	6	2	4	..	6	..	..	6	2	10	..	..	..
B. leprae—Kedrowsky	2.0	0.1	0.01	30	2	1	8	10	4	30	6	4	**0.2**	0.4	1	1	1	1	8	4	2	0.6	4	4	30	0
B. leprae—Duval (nonchrome)	1.0	0.1	0.02	30	2	1	8	8	4	30	6	4	0.2	**0.2**	1	1	1	1	10	6	2	0.6	4	4	..	..
B. tuberculosis—human	1.0	0.1	0.01	30	4	1	30	8	4	30	6	4	0.2	1	**0.6**	0.6	1	1	10	6	4	1	4	4	30	0
B. tuberculosis—bovine	0.8	0.1	....	30	2	2	30	10	8	30	8	4	1	4	0.6	**0.6**	1	1	30	6	6	1	30	..	30	0
B. tuberculosis—avian	0.3	0.05	....	100	1	4	8	30	10	50	10	30	1	1	4	2	**1**	2	8	4	2	4	6	..	10	0
B. tuberculosis—turtle	0.6	0.05	....	100	1	4	10	30	10	0	..	30	4	4	6	6	8	**1**	8	8	4	1	6	..	30	0
B. of dung—Moeller	0.3	0.05	....	100	0.6	6	6	30	8	50	..	30	4	6	6	2	4	2	**1**	2	0.6	2	4	..	10	0
B. leprae—Karlinsky	0.4	0.05	....	50	1	6	8	30	8	100	..	30	4	4	6	2	8	2	1	**2**	1	1	4	30	10	0
B. of timothy—Moeller	0.2	0.05	....	100	1	10	8	30	10	100	..	30	4	4	6	2	6	4	1	2	**0.3**	2	6	10	30	0
B. of butter	0.6	0.1	....	30	2	4	30	10	10	30	30	6	2	4	2	4	6	2	10	6	2	**2**	6	100	..	..
B. smegmatis	0.6	0.1	0.04	10	1	2	8	8	4	30	6	4	1	4	2	1	4	1	8	4	2	0.6	**1**	6	30	0
B. typhosus	0.3	0.05	....	0	8	0	0	0	50	0	0	0	30	50	30	100	30	30	30	30	10	0	0	**0.4**	30	0
B. coli	0.2	0.05	....	0	8	0	9	0	0	0	0	0	30	0	30	30	10	30	30	10	10	0	0	..	30	0
B. abortus	0.2	0.05	....	..	..	..	0	0	0	..	..	..	0	10	0	50	0	..	..	0	..	0	..	10	..	..
B. diphtheroid	0.3	0.05	0.02	..	8	..	0	100	40	0	0	0	30	..	30	30	..	30	0	..	30	..	..	30	**0.6**	**6**

0 means no fixation with 0.2 c c.

number of antigens will give fixation with that serum near its titer limit with the homologous antigen. In other words, when a serum is titrated with a number of antigens, some will give fixation with a relatively small amount of the serum, while other antigens will fix only with considerably larger amounts. In order to make this more evident, the cross-fixations have been slightly rearranged in table 4. Here are shown only those antigens giving fixation with amounts of serum near the titer with the homologous antigen. The inclusion of only those antigens reacting with 5 times the minimal fixing dose of each serum with its homologous antigen has been arbitrarily made. The figures in the table indicate the multiples of that dose of serum necessary for fixation with any given antigen, e. g., 1 = the minimal fixing dose of serum for the homologous antigen, 2 = twice that amount, etc. B. leprae 18 has been omitted because of its poor antigenic properties.

In the tables a general group reaction including all the acid-fast organisms studied is conspicuous. Although, as has been pointed out, a given immune serum may fix well with only a moderate number of the antigens, and less well with the remainder, yet practically all the antigens give good fixation with a considerable number of the serums. Indeed, despite a few irregularities which will be discussed later, this group reaction appears sufficiently widespread and specific to deserve the name acid-fast fixation.

The attempt to classify the various organisms by grouping those antigens which gave the best fixation with the several serums has been made in table 4. Although an accurate differentiation and grouping of the acid-fast bacilli is rendered difficult by the general fixation affecting all members, nevertheless, a certain tendency to form subgroups can be detected clearly. This tendency is more striking in some cases than in others. The group of chromogenic leprosy bacilli, composed of the Duval,[11] Levy,[14] B, F, G and H organisms, has a definite similarity in antigenic characters as shown by the cross-fixations both with the immune serums and antigens. This antigenic relationship is so well defined with the F, G and H strains that one is tempted to consider these organisms identical. It is significant that all three were isolated by the same observer (Clegg), two being from the Philippine Islands and the third from Hawaii. The Duval, Levy and B organisms seem to be more closely related to each other antigenically than to the other three strains. The remaining two chromo-

TABLE 4

Fixation Reaction with Immune Rabbit Seriums and Various Antigens Showing Antigens Reacting with Five Times The Minimal Fixing Dose of Each Serum of Less

	Immune Rabbit Serums																				
	B. leprae—Duval (chrome)	B. leprae—Levy	B. leprae—B	B. leprae—F	B. leprae—G	B. leprae—H	B. leprae—Hansen	B. smegmatis	B. leprae—Kedrowsky	B. leprae—Duval (nonchrome)	B. tuberculosis—Human	B. tuberculosis—Bovine	B. tuberculosis—Avian	B. tuberculosis—Turtle	B. of dung	B. leprae—Karlinsky	B. of timothy	B. of butter	B. leprae—Bayon	B. typhosus	B. diphtheroid, Serum 1	B. diphtheroid, Serum 2
B. leprae—Duval (chrome)	1	4	..	..	..	..	2.5	..	..	..	4	2	4	1	..	3	..	..	4	..	..	
B. leprae—Levy	2	1	4	..	..	..	2	..	..	..	4	4	..	4	..	..	..	..	..	..	..	
B. leprae—B	1	2	1	..	..	..	2	4	..	..	..	2	2	2	..	..	..	2	..	..	..	
B. leprae—F	4	4	..	1	2	2	..	..	..	..	..	2	4	4	..	..	..	..	..	..	..	
B. leprae—G	3	4	4	1	1	2	..	..	..	..	..	4	..	..	..	..	..	..	..	..	..	..
B. leprae—H	..	..	..	1	1	1	..	..	..	..	..	2	..	..	..	..	..	..	..	..	..	..
B. leprae—Hansen (from skin)	..	..	..	..	..	..	1	..	..	..	2	..	..	..	..	..	..	4	..	..	..	..
B. smegmatis	5	2	..	..	4	..	1	1	3	..	4	2	4	1	..	2	..	1	2	..	..	..
B. leprae—Kedrowsky	..	1	..	..	4	..	1	4	1	2	2	2	1	1	..	2	..	1	4	..	..	..
B. leprae—Duval (nonchrome)	..	1	..	..	4	..	1	4	1	1	1	2	1	1	..	3	..	1	4	..	..	..
B. tuberculosis—human	..	1	..	..	4	..	1	4	1	5	1	1	1	1	..	3	..	2	..	..	..	..
B. tuberculosis—bovine	..	2	..	..	..	..	1	..	3	..	..	1	1	1	..	3	..	2	4	..	..	..
B. tuberculosis—avian	..	4	..	..	..	..	..	..	3	5	..	4	1	2	..	2	..	..	2	..	..	..
B. tuberculosis—turtle	..	4	..	..	..	..	..	..	..	..	..	..	..	1	..	4	..	2	2	..	..	..
B. of dung—Moeller	..	..	..	..	..	..	..	4	..	..	..	4	4	2	1	1	2	4	1	..	..	..
B. leprae—Karlinsky	..	..	..	..	..	..	..	4	..	..	..	4	..	2	1	1	3	2	2	..	..	..
B. of timothy—Moeller	..	..	..	..	..	..	..	..	..	..	..	4	..	4	1	1	1	4	2	..	..	..
B. of butter	..	4	..	..	..	..	1.5	..	.	..	4	..	..	2	..	3	..	4	4	..	..	..
B. leprae—Bayon	..	..	..	..	..	..	..	..	..	..	..	4	..	1	4	2	3	4	1	..	..	..
B. typhosus	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	1	..	..
B. diphtheroid	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	1	1

genic leprosy cultures studied appear unrelated to the others, one of them, 18, having very poor antigenic qualities and the other, Bayon's culture, falling in another group.

The remaining organisms form a large group distinct from the organisms just mentioned, but even here the tendency to form subgroups can be detected. There is a somewhat more marked cross-fixation with those strains given last in the table (table 4) including the bovine, avian, and turtle tubercle bacilli, dung, timothy and butter bacilli, and the leprosy strains of Karlinsky[15] and Bayon. Although this group is perhaps less well defined than the one including the chromogenic leprosy bacilli, their antigenic relationship seems distinct. Of the other organisms, four strains have the striking property of fixing well with a majority of the immune serums. These antigens are the nonchromogenic B. leprae cultures of Duval[11] and Kedrowsky,[9] B. tuberculosis (human) and the smegma bacillus. This characteristic of the latter organisms is associated with a physical property of the antigens themselves, since these particular antigens could be suspended in salt solution better than the others. They gave a uniform antigen suspension free from clumps, and the bacterial protein appeared as an evenly distributed opacity. With the other antigens this even fine distribution of the antigen in suspension was disturbed by some coarser flocculi which it seemed impossible to break up, although, for the most part, the suspensions appeared finely divided. This inability to secure a uniformly even distribution of the antigen suspension was attributed to the fatty substances of the organisms. One strain, B. leprae 18, which seemed especially rich in waxy material, showed a constant tendency to form numerous coarse particles in the antigen suspension, and it is of interest that this organism showed the poorest antigenic properties of any of the acid-fast bacilli studied. In general, it was found that the organisms which gave the most even suspensions made the best antigens. The Hansen bacilli obtained from leper skin, while conforming to the general acid-fast fixation of the rest of the organisms, does not appear closely related to the cultures. Possibly certain other antigenic relationships may be indicated from the reactions, but they are somewhat indefinite. Even in the sub-groups indicated there are irregularities in the results with occasionally unexpected cross-fixations. These are unexplained and are possibly an evidence of the group reaction.

The relation of the nonacid-fast organisms used as controls to the group of acid-fast antigens is striking. The bacteria used, B. typhosus, B. coli, B. abortus, and a diphtheroid bacillus were chosen merely as examples of common nonacid-fast organisms. The fact that diphtheroid bacilli have been isolated from lepers and are considered by some observers (Kedrowsky) related to the acid-fast leprosy organism, influenced the selection of a member of this group. The tests with these antigens show a very interesting and striking nonspecific fixation. Many of the rabbit serums showed fixation with one or more of the nonacid-fast antigens, but always with relatively large amounts of serum. This difference in titer of a serum with the nonacid-fast and the acid-fast antigens, particularly that of the homologous organism, is very marked. Usually from 30 to several hundred times the minimal fixing dose of a serum with the homologous antigen is necessary to obtain fixation with a nonacid-fast strain. Also, all serums which gave fixation with the nonacid-fast organisms were of relatively high titer while the low titer serums failed to give such fixation.

That this nonspecific fixation is a rather general phenomenon is indicated by the fact that, not only can fixation be obtained with nonacid-fast antigens and rabbit serums highly immunized against acid-fast strains, but a highly immune antityphoid serum or antidiphtheroid serum will also give fixation with acid-fast and other antigens. The antityphoid serum tested in table 3 had an agglutination titer of 1: 10,000 and gave fixation with all other antigens tested, although only in relatively low dilutions. A good example of the effect of the titer of an immune serum on nonspecific fixation with other antigens, is given in the results of two serums of different titer from rabbits immunized with the same antigen. In table 3 are shown the results of fixation tests with two serums immunized with a diphtheroid bacillus, one having a titer of 0.006 c c with the homologous antigen, and the other of 0.0006 c c. With the high titer serum, a nonspecific fixation is obtained with a number of heterologous antigens, although in only relatively low dilution, while with the serum of lower titer, fixation is not shown with other antigens. In every case nonspecific fixation occurs only with the serums of relatively high titer, and with many times the minimal fixing dose of such serums with the homologous antigens.

In the presence of such nonspecific fixation with bacterial antigens, the question arises of the relation of this phenomenon to the group fixation with the acid-fast organisms, and whether this group reaction may not be another evidence of nonspecific fixation. From one point of view it is certainly nonspecific, since frequently the organisms of this group have marked cultural, pathogenic and other biologic differences, but it is likewise evident that, as a group reaction, it may be considered specific for this group of organisms as distinguished from the nonacid-fast strains. In certain instances, when an acid-fast antigen gives fixation with an immune serum only in rather low dilutions, it seems probable that such fixation is an example of the general nonspecific fixation. A very large proportion of the cross-fixations with the acid-fast antigens, however, show fixation with amounts of serum that approach the titer limit of the serums. This high degree of cross-fixation does not occur in the usual nonspecific fixation noted and indicates a group reaction distinct from the nonspecific variety.

The cause of nonspecific fixation such as has been illustrated in this series of experiments is not altogether clear. It seems certain that anticomplementary action of the antigens or immune serums can be excluded. Two units of complement with amounts of antigen and serum that were not anticomplementary in four times the dose used, together with the usual controls, seem to make this explanation impossible. The method of preparation of the antigens, with the possibility of the introduction of substances such as small amounts of culture mediums or products of culture autolysis, might conceivably be concerned in the production of a nonspecific fixation. This factor has been controlled by several experiments, unnecessary to give in detail, in which both acid-fast and nonacid-fast washed organisms were used as antigens with the immune serums in similar fixation tests. Several rabbit serums immunized with washed bacteria were also tested with the antigens used in the larger series. In all instances, no differences in the nonspecific fixation were noted that could be attributed to the method of antigen preparation. It seems likely, therefore, that the bacterial antigens themselves contain the substances that are concerned in the production of the nonspecific fixation.

The tendency of immune serums of high titer to give such nonspecific fixation with other antigens as has been mentioned suggests that a certain amount of care should be used in all cross-fixations with high titer serums to avoid being misled by the general reaction. It is felt that the somewhat more delicate method of performing the tests used in these experiments has made nonspecific fixation more apparent, but there is no reason to believe that it could not be demonstrated by other methods with the amounts of serum ordinarily used, e. g., 0.1 c c.

That the part played by the lipoid material of the acid-fast organisms in the fixation test may be important is indicated by the fact that ether extracts of tubercle bacilli and other acid-fasts may act as antigen in tests with immune serums, or with serums from patients suffering from such acid-fast infections as tuberculosis and leprosy. The work of Much[3, 4] and his collaborators[5, 6] with the so-called "partial antigens," while rather unconvincing in many respects, nevertheless illustrates this phenomenon. Lucke's[19] experiments with tubercle wax antigen, also, show that the lipoid material from tubercle bacilli acts as antigen in fixation tests with serums of certain tuberculous patients. All such experiments have shown a rather high percentage of nonspecific fixations, possibly due to the relatively large dose of antigen employed in the tests. Such antigens are much inferior also to whole culture antigens in the number of positive tests obtained with serums of tuberculous individuals. In the present work no study of such antigens was made except to show that ether extracts of several dried antigens gave fixation with some of the immune rabbit serums. It is believed by some writers that fixation with such antigens is due merely to general lipotropic properties in certain serums since a rather large proportion of cases of syphilis give a positive reaction. None of the rabbit serums used in the present experiments, however, showed fixation even in amounts of 0.2 c c with a simple lipoid (Wassermann) antigen. With the present evidence it does not seem justifiable to regard the fixation test with immune rabbit serums and acid-fast antigens a purely lipotropic phenomenon. The presence of relatively large amounts of waxy or lipoidal material is one of the striking characteristics common to all the members of the acid-fast group, and it is reasonable to suppose that they are concerned in the group reaction. Even assuming that their part in the process is essential and that the fixation depends on their presence does not alter the specificity of the reaction for this group since other bacteria contain proportionately small amounts of lipoid material. It seem appropriate, therefore, to employ the term acid-fast fixation.

---

[19] J. Immunol., 1916, 1, p. 457.

Another possible explanation for such nonspecific fixation is that it is an evidence of a general sensitization to bacterial protein which occurs in serums of highly immunized animals. Such a sensitization might even include any foreign protein. Since no effort has been made in these experiments to demonstrate a general sensitization of this nature, its possibility may be merely mentioned.

SUMMARY

In serums of rabbits immunized with various acid-fast bacteria there is present a complement-fixing antibody which reacts with all members of the acid-fast group examined. When such antibodies are studied quantitatively by titrating each immune serum with the different antigens in the fixation test, certain members of the group show a close antigenic relationship, and tend to form subgroups. Certain organisms of the group are less closely related, but cross-fixation is specific for this group of bacteria and may be termed an acid-fast fixation.

Some of the acid-fast bacterial antigens are constantly superior to others in giving fixation with high dilutions of all antiserums obtained with members of this group, and these antigens are those that form even fine suspensions in salt solution. Those antigens with much waxy material tend to form suspensions containing coarser particles, and have inferior antigenic properties.

Control nonacid-fast bacterial antigens and rabbit serums immunized with such organisms were compared with the acid-fast antigens and their immune serums. Nonspecific fixation is obtained with nonacid-fast antigens in rabbit serums of high fixation titer immunized with acid-fast strains. Such fixation, however, is found only with relatively large amounts of antigen or serum. Similarly, acid-fast antigens give fixation in low dilutions only, if at all, with immune serums from nonacid-fast strains. This nonspecific fixation occurs only in immune serums of high titer, and, when present, is obtained only with many times the minimal fixing dose of serum with the homologous antigen. It is distinct from the group acid-fast fixation, and may be an evidence of sensitization to bacterial proteins in all highly immunized serums.